Investigating the Potential for Cryopreservation of Human Granulocytes with Concentrated Glycerol

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Abstract

The purpose of this study was to investigate the potential for cryopreservation of granulocytes using 30% glycerol. Recently reported permeability data was used to design two different methods for addition and removal of glycerol: a fast method that is predicted to keep cell volumes between 80% and 150% of the isotonic volume and a slow method that is predicted to keep cell volumes between 80% and 115% of the isotonic volume. The fast method resulted in cell recoveries of $31\% \pm 9\%$ and $11\% \pm 3\%$ before and after freezing, respectively, whereas the slow method resulted in even lower cell recoveries of $5\% \pm 2\%$ and $4\% \pm 2\%$. The reduced cell recovery for the slow method is consistent with an increase in damage as a result of glycerol toxicity. Our results suggest that cryopreservation of granulocytes in concentrated glycerol is not feasible.

Keywords: polymorphonuclear cells, osmotic damage, cryoprotectant, toxicity, transfusion

Increasing interest in granulocyte transfusions for neutropenic patients, antibiotic resistant infections, and patients with weakened immune systems due to chemotherapy have led to an increased demand for granulocytes. Unfortunately, granulocytes have a very short shelf life of less than 24 hours. To increase the availability of granulocytes for transfusion, cryopreservation or another method of long-term storage is necessary. Granulocyte cryopreservation has long eluded scientists despite successes with many other types of cells. Dimethyl sulfoxide has been the cryoprotective agent (CPA) of choice for granulocytes. While success has been demonstrated in some cases, the results are very inconsistent, and there is no consensus method [2,10]. Dimethyl sulfoxide also has negative side effects when administered clinically. Glycerol is a logical choice as a CPA since it has been used in high concentrations to successfully preserve red blood cells. Cryopreservation of granulocytes with glycerol has been attempted before and failed consistently. Some have suspected osmotic damage as the culprit [6,11]. Others suspect toxicity as the leading obstacle to successful cryopreservation with glycerol [1,7]. Much of the trouble with designing an accurate experiment lies in predicting cell volume changes. Until recently, glycerol permeability data was limited to two studies which reported vastly different permeability values [5,7].

The objective of this study was to design a convenient method for loading concentrated glycerol into granulocytes while limiting osmotic damage. Glycerol permeability values were based upon the most recent research, using modern equipment [12]. We examined two different approaches for equilibrating granulocytes with glycerol, a fast method (Fig. 1) and a slow method (Fig. 2). The starting point for the fast method was the plot in Vian and Higgins [12] for a two-step addition process, in which the cells are first exposed to a ~ 1 molal glycerol loading solution containing a hypotonic concentration of nonpermeating solutes. The cells are predicted

to shrink to about 80% of their isotonic volume in this first step, followed by swelling to about 150% of the isotonic volume (Fig. 1). In the second step the cells are exposed to 30% w/v glycerol, which causes shrinkage to about 80% of the isotonic volume. A three-step removal process was used to prevent excessive swelling; this procedure is predicted to keep the cell volumes below 150% of the isotonic volume (Fig. 1).

The slow glycerol equilibration method consisted of 4 addition steps and 4 removal steps, and is predicted to maintain cell volumes between 80% and 115% of the isotonic volume (Fig. 2). Compared with the fast method, this approach is expected to result in less extreme volume changes, but requires more steps and is more time consuming.

Granulocytes were isolated from whole blood following a procedure similar to our previous study [12]. Whole blood was collected from volunteer donors using an IRB approved protocol, layered on top of an equal volume of Polymorphprep (Cosmo Bio USA, Carlsbad, CA, USA), and then centrifuged to separate the cells into distinct layers. The granulocyte layer was transferred into a 50 mL centrifuge tube, washed with HBSS free and pelleted. The pellet was resuspended in 5 mL of ACK Red Blood Cell Lysis Buffer (150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) to lyse any contaminating red blood cells [9]. After sitting 5 minutes, the tube was again filled with HBSS free and centrifuged. The lysis process was repeated once more, letting the cells sit 3 minutes in ACK. One final fill with HBSS and centrifugation resulted in a pellet of pure granulocytes. This pellet was resuspended in 300 μL of granulocyte buffer (isotonic HBSS free containing 1% w/v BSA and 0.1% w/v EDTA). Over 95% of the cells in the resulting suspension had intact membranes, as assessed by trypan blue exclusion.

Following granulocyte isolation and assessment of membrane integrity, aliquots of 100 μ l of concentrated cell suspension were prepared for glycerol addition, freezing, thawing and glycerol removal. Freezing was carried out in 2 mL cryovials using a Mr. Frosty[®] freezing container, which produces a cooling rate of about 1 $^{\circ}$ C/min. After storage for two hours in a -80 $^{\circ}$ C freezer, samples were placed in the vapor phase of liquid nitrogen for 30 minutes prior to thawing in a 37 $^{\circ}$ C water bath.

While the fast glycerol addition and removal procedure is predicted to maintain cell volumes within the osmotic tolerance limits of granulocytes (Fig. 1), it didn't yield a particularly high recovery of membrane-intact cells, as shown in Fig. 3. Glycerol addition and removal resulted in an average intact cell recovery of $31\% \pm 9\%$ compared to the number of membrane-intact cells in the original cell suspension. When cells subjected to the fast glycerol equilibration method were frozen and thawed, the intact cell recovery was only $11\% \pm 3\%$ compared to the original cell suspension. We also examined the effects of fast glycerol equilibration on membrane integrity by comparing the number of intact cells after treatment to the total number of cells present in the sample. Of those cells present after fast glycerol equilibration, just over half had intact membranes ($57\% \pm 9\%$ without freezing and thawing and $56\% \pm 4\%$ with freezing and thawing). These results are summarized in Table 1.

We hypothesized that these cell losses may have been caused by the nonconventional glycerol loading process, in which the cells were exposed to glycerol in a hypotonic carrier solution that has the potential to induce excessive swelling. Therefore, we also examined a more conventional, slow procedure involving exposure to glycerol in isotonic carrier solution and additional steps to reduce the extent of cell swelling (see Fig. 2). However, the slow glycerol approach resulted in even lower recovery of membrane-intact cells ($5\% \pm 2\%$ without freezing

and thawing and $4\% \pm 2\%$ with freezing and thawing), and the majority of the cells present after slow glycerol equilibration had compromised membranes (see Table 1). To rule out the potential for damage caused by sample handling, we also performed a control experiment in which the sample was subjected to the same handling as the slow glycerol method (including centrifugation), but using isotonic saline as the diluent. The handling control yielded an average intact cell recovery of $78\% \pm 8\%$ compared to the original cell suspension, and over 98% of the cells present in the sample after handling had intact membranes.

Taken together, our results suggest that glycerol may be toxic to granulocytes. The slow glycerol equilibration method is gentler osmotically (keeping predicted volumes between 80% and 115% of the isotonic volume), but results in more prolonged exposure to concentrated glycerol solutions. This slow glycerol method resulted in significantly lower cell recovery than the fast method, pointing to toxicity as the mechanism of damage.

Interestingly, the fast glycerol equilibration method is similar to procedures designed to be minimally toxic based on minimization of a CPA toxicity cost function [3,4]. These minimally toxic procedures also involve CPA loading using a hypotonic carrier solution that induces cell swelling. Swelling is potentially advantageous for reducing toxicity because it enables the use of a lower (and hence less toxic) CPA concentration to load a given amount of CPA into the cell. Our rationale for the fast glycerol method was to reduce the number of required steps by taking advantage of the full range of tolerable cell volumes, similar to the approach described by Meryman [8]. The resulting two-step addition procedure may have also had the added advantage of reducing CPA toxicity.

The literature provides conflicting evidence about the toxicity of glycerol to the human granulocyte. Dooley and colleagues used a cross flow filtration system for gradual addition and removal of glycerol and reported that granulocytes could tolerate exposure to up to 3.7 mol/L glycerol with little loss of chemotactic activity [6]. In contrast, substantial cell losses have been observed after exposure to glycerol concentrations as low as 0.5-1 mol/L using procedures that were predicted to be nondamaging osmotically [1,7], suggesting that glycerol may be toxic. Takahashi and colleagues provide the most thorough investigation of the effects of glycerol on human granulocytes [11]. While the authors were hesitant to conclude that glycerol was toxic, their results seem to indicate that this may be the case. A sharp decrease in cell recovery, phagocytosis and chemotactic activity occurred when granulocytes were exposed to 2 mol/L glycerol for more than an hour [11]. In addition, despite slow addition and removal to mitigate osmotic damage, the authors observed substantial loss of chemotactic activity after exposure to a glycerol concentration of 3 mol/L and complete loss of chemotactic activity for a glycerol concentration of 4 mol/L. Nonetheless, it is difficult to conclusively rule out osmotic damage. Takahashi et al obtained better results for slow addition and removal than rapid addition and removal and it is possible that their “slow” method was not slow enough to completely prevent osmotic damage. Our experiments provide new insight into the toxicity of glycerol. We compared slow and fast methods for glycerol equilibration, both of which were designed to avoid osmotic damage, and found that the fast method yielded better results than the slow method. It is unlikely that these results can be explained in terms of osmotic damage, as the slow method is predicted to result in less swelling than the fast method (see Figs. 1 and 2). Our results are, however, consistent with increased toxicity caused by prolonged exposure to high glycerol concentrations during the slow method. These results provide further support for the hypothesis

135 that glycerol is toxic to granulocytes, and suggest that cryopreservation of granulocytes in
136 concentrated glycerol is not feasible.

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Figure Captions

Fig. 1. Fast method for equilibration of granulocytes in 30% w/v glycerol. All diluent solutions were pre-equilibrated in a 37 °C water bath.

Fig. 2. Slow method for equilibration of granulocytes in 30% w/v glycerol. All diluent solutions were pre-equilibrated in a 37 °C water bath. Solution A consists of 4.75 Osm/kg glycerol in hypertonic (0.45 Osm/kg) HBSS free. Solution B consists of hypertonic (0.45 Osm/kg) HBSS free. Centrifugation was carried out at 250 g for 10 min.

Fig. 3. Effects of glycerol addition/removal and freezing/thawing on recovery of granulocytes with intact membranes. Cells were equilibrated with 30% w/v glycerol using either a fast method (Fig. 1) or a slow method (Fig. 2). For comparison, the cells were also subjected to the same handling as in the slow glycerol method, but using isotonic buffer only. Bars denoted with distinct letters represent significantly different recoveries of membrane-intact cells ($p < 0.05$, Fisher's Least Significant Difference tests, $n = 4-6$). Error bars represent the standard error of the mean.

References

- [1] W.J. Armitage, P. Mazur, Toxic and osmotic effects of glycerol on human granulocytes, *Am J Physiol* 247 (1984) C382-389.
- [2] H. Bank, Granulocyte preservation circa 1980, *Cryobiology* 17 (1980) 187-197.
- [3] A.F. Davidson, J.D. Benson, A.Z. Higgins, Mathematically optimized cryoprotectant equilibration procedures for cryopreservation of human oocytes, *Theoretical Biology and Medical Modelling* 11 (2014).
- [4] A.F. Davidson, C. Glasscock, D.R. McClanahan, J.D. Benson, A.Z. Higgins, Toxicity Minimized Cryoprotectant Addition and Removal Procedures for Adherent Endothelial Cells, *PLoS ONE* 10 (2015) e0142828.
- [5] D.C. Dooley, Glycerol permeation of the human granulocyte, *Experimental Hematology* 10 (1982) 413-422.
- [6] D.C. Dooley, P. Law, P. Schork, H.T. Meryman, Glycerolization of the human neutrophil for cryopreservation: osmotic response of the cell, *Experimental Hematology* 10 (1982) 423-434.
- [7] J. Frim, P. Mazur, Interactions of cooling rate, warming rate, glycerol concentration, and dilution procedure on the viability of frozen-thawed human granulocytes, *Cryobiology* 20 (1983) 657-676.
- [8] H.T. Meryman, Cryopreservation of living cells: principles and practice, *Transfusion* 47 (2007) 935-945.
- [9] H. Oh, B. Siano, S. Diamond, Neutrophil isolation protocol, *J Vis Exp* (2008).
- [10] A. Sputtek, P. Kuhn, A.W. Rowe, Cryopreservation of erythrocytes, thrombocytes, and lymphocytes, *Transfusion Medicine and Hemotherapy* 34 (2007) 262-267.

187 [11] T. Takahashi, J.B. Bross, R.E. Shaber, R.J. Williams, Effect of cryoprotectants on the
188 viability and function of unfrozen human polymorphonuclear cells, *Cryobiology* 22
189 (1985) 336-350.

190 [12] A.M. Vian, A.Z. Higgins, Membrane permeability of the human granulocyte to water,
191 dimethyl sulfoxide, glycerol, propylene glycol and ethylene glycol, *Cryobiology* 68
192 (2014) 35-42.

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Figure 1

Figure 2

Figure 3

Table 1. Effects of glycerol addition/removal and freezing/thawing on membrane integrity.

Experimental Treatment	Recovery of membrane intact cells compared to original cell suspension ^a	Percentage of cells in treated sample with an intact cell membrane ^b
Fast glycerol addition/removal	31% ± 9%	57% ± 9%
Fast glycerol addition/removal + freeze/thaw	11% ± 3%	56% ± 4%
Slow glycerol addition/removal	5% ± 2%	20% ± 4%
Slow glycerol addition/removal + freeze/thaw	4% ± 2%	35% ± 7%
Handling control	78% ± 8%	98.8% ± 0.3%

^a 100% x $\left(\frac{\text{membrane intact cells after treatment}}{\text{membrane intact cells before treatment}} \right)$

^b 100% x $\left(\frac{\text{membrane intact cells after treatment}}{\text{total cells after treatment}} \right)$